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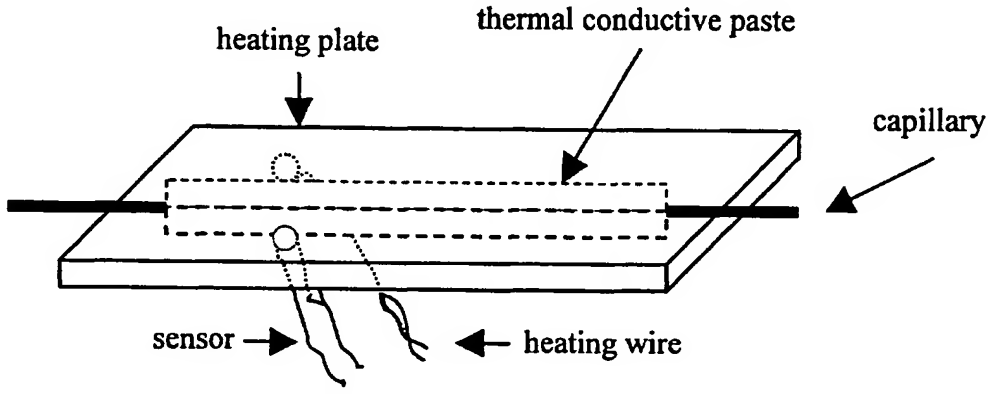

(57) Abstract: A method for detecting the presence of mutations in DNA samples by multiplexed electrophoresis, which method comprises: (a) introducing each of the DNA samples, all of which comprise a buffer solution comprising a matrix and at least one of which comprises heteroduplexed DNA, separately into a plurality of containers in an electrophoresis system; (b) heating the DNA samples independently of the electrical field in the electrophoresis system through at least one predetermined spatial or temporal temperature range selected to denature the DNA samples; and (c) detecting the presence of any mutations in the DNA samples by comparing an electrophoretic pattern for each of the DNA samples with an electrophoretic pattern for a control DNA sample, whereupon the presence of mutations in DNA samples is detected by multiplexed electrophoresis. The method can further comprise in (b) introducing at least one control DNA sample, which comprises a homoduplexed DNA in a buffer solution comprising a matrix, separately into a container in the plurality of containers in the electrophoresis system.

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HIGH-THROUGHPUT DETECTION OF UNKNOWN DNA MUTATIONS BY USING MULTIPLEXED CAPILLARY ELECTROPHORESIS

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This application claims priority to co-pending U.S. provisional patent application no. 60/194,597, which was filed on April 5, 2000.

GOVERNMENT RIGHTS

This invention was made with government support under Contract No. W-7405-Eng-82 awarded by the U.S. Department of Energy and HHS Contract No. HG01804 awarded by the National Institutes of Health. The Government may have certain rights in this invention.

TECHNICAL FIELD OF THE INVENTION

This invention relates to the use of multiplexed capillary electrophoresis to detect unknown DNA mutations in a high-throughput manner.

BACKGROUND OF THE INVENTION

Detection of mutations in DNA has been increasingly important in the fields of genetics, molecular diagnostics, and cancer research. Single-nucleotide polymorphism (SNP) is the most common form of genetic variation and is the most difficult form to detect. This type of single-based substitution in the genome occurs at a frequency in excess of 1% in the human population. Other types of mutations involve insertion and deletion. In general, mutations can be used to detect genetic linkages and to diagnose diseases, such as cancer.

Of course, the ultimate method of detecting a DNA mutation is by sequencing the DNA. However, current DNA sequencing techniques are still relatively laborious and expensive. Even further, large-scale DNA sequencing to detect mutations is also considered to be inefficient because a large portion of the DNA that is sequenced does not comprise a mutation. Accordingly, rapid screening methods are needed to enable the detection of known point mutations and unknown point mutations before any further characterization of the DNA (such as complete sequencing) is undertaken.

Several different analytical techniques are known, and some have been suggested for the detection of DNA mutations. One technique is temperature-gradient gel electrophoresis (TGGE). In TGGE a spatial or temporal temperature gradient is used to provide various degrees of denaturation along the course of

DNA separation. Another known technique is temperature-gradient capillary electrophoresis (TGCE), which involves generating a temperature sweep internally via ohmic heat produced by a voltage ramp over time. This apparatus does not allow the temperature and the electric field to be separately controlled. This is a serious limitation in capillary arrays where a common voltage source is employed. In addition, inherent differences in dimensions or separation matrices among the capillaries within the array, for example, will result in variations in the temperature profiles as will the heat generated by each capillary affect the temperature of other capillaries in the absence of active control.

Another known method for detecting DNA mutations is denaturing high performance liquid chromatography (DHPLC) as described by Oefner et al. (Am. J. Hum. Genet. 57: A266 (1995)). Heteroduplexes and homoduplexes formed as a result of the presence of mutation can be separated in an HPLC column based on differences in conformation or hydrophobicity. HPLC, however, requires expensive specialized columns and utilizes large volumes of solvents and samples. HPLC also is not suitable for parallel operation in a highly multiplexed mode. In the scheme proposed by Oefner et al., it is further necessary to predetermine the optimal operational temperature for separation of heteroduplexes and homoduplexes for each DNA sample. Thus, it is not applicable to detecting unknown DNA mutations.

In view of the above, it is an object of the present invention to provide a method of detecting known and unknown mutations in DNA in a high-throughput, high-detection-rate, and low-cost manner. This and other objects and advantages of the present invention, as well as additional inventive features, will become apparent in the detailed description of the present invention provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method for detecting the presence of mutations in DNA samples by multiplexed electrophoresis. The method comprises:

(a) introducing each of the DNA samples, all of which comprise a buffer solution comprising a matrix and at least one of which comprises heteroduplexed DNA, separately into a plurality of containers in an electrophoresis system;

(b) heating the DNA samples independently of the electrical field in the electrophoresis system through at least one predetermined spatial or temporal temperature range selected to denature the DNA samples; and

(c) detecting the presence of any mutations in the DNA samples by comparing an electrophoretic pattern for each of the DNA samples with an electrophoretic pattern for a control DNA sample, whereupon the presence of mutations in the DNA samples is detected by multiplexed electrophoresis. The method can further comprise in (b) introducing at least one control DNA sample, which comprises a homoduplexed DNA in a buffer solution comprising a matrix, separately into a container in the plurality of containers in the electrophoresis system.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a photograph of a system for use in the present inventive method.

Fig. 2 is a diagram of the heating device for temperature-gradient capillary electrophoresis.

Fig. 3A is the calculated melt map (melting temperature (Tm) (°C) vs. position (base pairs (bp)) of sample M2 of Table 1.

Fig. 3B is the calculated melt map (Tm (°C) vs. position (bp)) of sample M60 of Table 1.

Fig. 3C is the calculated melt map (Tm (°C) vs. position (bp)) of sample M69 of Table 1.

Fig. 3D is the calculated melt map (Tm (°C) vs. position (bp)) of sample M122 of Table 1.

Fig. 4A is the electropherogram (intensity vs. time (min)) of sample M2 of Table 1 at a temperature gradient of 61-71 °C and a ramp of 0.7 °C/min.

Fig. 4B is the electropherogram (intensity vs. time (min)) of sample M60 of Table 1 at a temperature gradient of 61-71 °C and a ramp of 0.7 °C/min.

Fig. 4C is the electropherogram (intensity vs. time (min)) of sample M69 of Table 1 at a temperature gradient of 61-71 °C and a ramp of 0.7 °C/min.

Fig. 4D is the electropherogram (intensity vs. time (min)) of sample M122(I) of Table 1 at a temperature gradient of 61-71 °C and a ramp of 0.7 °C/min.

Fig. 4E is the electropherogram (intensity vs. time (min)) of sample M122(II) of Table 1 at a temperature gradient of 61-71 °C and a ramp of 0.5 °C/min.

Fig. 5A is the electropherogram (intensity vs. time (min)) of sample M60 of Table 1 at a temperature gradient of 68-74 °C and a ramp of 0.5 °C/min.

Fig. 5B is the electropherogram (intensity vs. time (min)) of sample M60 of Table 1 at a temperature gradient of 61-68 °C and a ramp of 0.5 °C/min.

Fig. 6 is a two-dimensional image (migration time vs. number of the capillary for time scale of 0-40 min) of the 96 electropherograms for detection of mutations in the five heteroduplex and four homoduplex fragments.

Fig. 7 represents extracted electropherograms (intensity vs. time (sec)), one for each of the mutation samples studied, from Fig. 6.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention provides an improved system and method for the high-throughput detection of unknown mutations by using multiplexed electrophoresis, such as multiplexed capillary electrophoresis. In general, any capillary array electrophoresis system can be used. An example of such a suitable system is described in Gao et al., Electrophoresis 20: 1518-1526 (1999), and Gong et al., Anal. Chem. 71: 4989-4996 (1999). Other suitable capillary array electrophoresis instrumentation is described in U.S. Patents 5,324,421; 5,498,324; 5,582,705; 5,695,626; and 5,741,411 to Yeung et al.

According to a principal aspect of the present invention, a reproducible temperature profile, independent of the electrical field involved, is provided so that an arbitrary temperature gradient can be selected. In general concept, the application of an elevated temperature partially denatures the DNA. If the DNA has a mismatch due to a mutation, the DNA is heteroduplexed and will start to melt at a lower temperature than a corresponding homoduplexed DNA, i.e., DNA without a mismatch due to mutation. The heteroduplexed DNA will, thus, exhibit a retarded migration behavior nearer the melting temperature as compared to that of the homoduplexed DNA in a sieving medium, such as a gel or a long-chain linear polymer solution. The mutation can be identified by the difference in electrophoretic patterns between homoduplexed and heteroduplexed DNA. We also note that homoduplexed DNA and heteroduplexed DNA possess different degrees of hydrophobicity, especially near the melting temperature. Therefore, other capillary electrophoresis principles can be applied in such instrumentation to screen for mutations.

The purpose of the present invention is to uncover as many mutations as possible without knowing the exact nature of the mutation. Thus, the presence of any mutation will lead to further evaluation.

In view of the above, the present invention provides a method for detecting the presence of mutations in DNA samples by multiplexed electrophoresis, particularly multiplexed capillary electrophoresis. The DNA samples can comprise DNA fragments, such as those generated by restriction-enzyme digestion

or polymerase chain reaction, for example. The DNA fragment samples, however, should be sufficiently purified and isolated and substantially free from degradation.

The method comprises:

(a) introducing each of the DNA samples, all of which comprise a buffer solution comprising a matrix and at least one of which comprises heteroduplexed DNA, separately into a plurality of containers in an electrophoresis system;

(b) heating the DNA samples independently of the electrical field in the electrophoresis system through at least one predetermined spatial or temporal temperature range selected to denature the DNA samples; and

(c) detecting the presence of any mutations in the DNA samples by comparing an electrophoretic pattern for each of the DNA samples with an electrophoretic pattern for a control DNA sample, whereupon the presence of mutations in the DNA samples is detected by multiplexed electrophoresis. The method can further comprise in (b) introducing at least one control DNA sample, which comprises a homoduplexed DNA in a buffer solution comprising a matrix, separately into a container in the plurality of containers in the electrophoresis system.

After (b), the denatured DNA samples desirably is allowed to reanneal if detecting in (c) will be by fluorescence; however, not all fluorophores require reannealing. If detecting in (c) will be by absorbance, then it is not necessary to allow the denatured DNA samples to reanneal.

Desirably, the heteroduplexed DNA is due to a mutation, which is preferably a point mutation, i.e., a substitution of one nucleotide for another. The mutation can be due to an insertion or deletion, which ultimately results in a frameshift mutation.

The buffer solution can be any suitable buffer. Desirably, the buffer is inert to the DNA sample, can maintain the working pH, and has suitable ionic strength for electrophoresis. The buffer concentration can be any suitable concentration in the range from 1-100 mM, for example. Examples of suitable buffers include, but are not limited to, 1xTBE, 50 mM phosphate, and 50 mM borate. Preferably, the buffer is 1xTBE. When a fluorophore is used, desirably the buffer is photobleached.

The buffer solution comprises any suitable matrix. Desirably, the sieving matrix has low fluorescence background and can interact specifically with the detectably labeled molecule to provide size-dependent retardation. The sieving

matrix can be present in any suitable concentration; from about 0.5% to about 10% is preferred. Similarly, a sieving matrix of any suitable molecular weight can be used; from about 100,000 to about 10 million is preferred. Examples of suitable matrices include, but are not limited to, poly(vinylpyrrolidone) (PVP), poly(ethylene oxide) (PEO), and hydroxyethylcellulose (HC). An especially preferred matrix is PVP, in particular PVP having an M_r of around 600,000. PVP has been successfully used in sequencing and genotyping and is further described in Gao et al. (1999), *supra*, and Yeung, Anal. Chem. 70: 1382-1390 (1998). PVP can separate DNA sequencing fragments and genotyping fragments up to at least 530 bp. In this regard, a 3% PVP solution enables good separation of DNA fragments, even though it is very dilute with a viscocity of less than 10 cP. Another advantage of PVP is that the same capillaries can be used for several months.

The buffer solution can further comprise a means of detecting the DNA, such as a fluorophore. Accordingly, examples of means of detecting the DNA include, but are not limited to, fluorescence, absorption and electrochemistry. If a fluorophore is used to detect the DNA, preferably, the fluorophore is an intercalating dye. The intercalating dye increases the rigidity of DNA and, thus, increases the efficiency of separation of DNA molecules. Preferably, there is enough intercalating dye present in the buffer such that at least one molecule of intercalating dye is present per 5 base pairs of DNA. Examples of intercalating dyes include ethidium bromide, Picogreen, POPO-III, TOTO-1 and YOYO-1. An especially preferred fluorophore is the intercalating dye ethidium bromide. All of the aforementioned dyes require that the denatured DNA be allowed to reanneal after heating and prior to detecting mutations.

When the means of detecting the DNA is a fluorophore, desirably it is one that is induced to fluoresce with a coherent light source, such as a laser, in which case a laser is used to induce the fluorescent label to fluoresce and the detecting includes determining the fluorescence of the DNA samples and, when present, the control samples. A preferred laser is an argon ion laser. The argon ion laser is preferably operated at 488 nm for detection of fluorescently labeled nucleic acid. The laser is focused at normal incidence to the sample. A lens, such as a lens having a focal length of 1.5 inch, can be used to focus the laser. If the laser generates extraneous light, an equilateral prism and at least one optical pinhole can be positioned before the imaging means and used to eliminate the extraneous light. The positioning of the equilateral prism and the optical pinhole is within the ordinary skill in the art.

Any suitable means can be used to introduce the DNA sample or the control DNA sample into a container in the context of the present inventive method. Preferred methods include pressure, gravity, vacuum, capillary or electrophoretic action.

Any suitable electrophoretic container can be used in the method. Desirably, the container is sufficiently small as to be conducive to detection of DNA molecules in a sample and is suitable for subjecting a sample contained therein to electrophoresis or hydrodynamic methods of sample introduction, such as pressure and gravity. Also desirably, the container allows the passage of light from a light source through the wall of the container facing the light source to the sample in the container. Thus, the walls of the container are desirably transparent, although, in some instances, the walls of the container can be translucent. In this regard, it is not necessary for the entirety of the walls of the container to allow the passage of light from the light source as described above as long as at least a portion of the walls of the container allow the passage of light from the light source such that the sample in the container is irradiated and absorption or fluorescence can be detected by the imaging means.

In general, the container should have smooth surfaces and uniformly thick walls and be made of a material that is transparent over the range of wavelengths of light that cause the detectably labeled molecule to absorb or fluoresce. Preferred materials for the containers include, but are not limited to, quartz, fused silica and glass. The cross-section of the container is not critical. Similarly, the thickness of the container is not critical. The wall should be of sufficient thickness so as to maintain the structural integrity of the container, yet not so thick as to impede adversely the passage of light through the container. The shape of the container also is not critical; the container can have any suitable shape.

A capillary tube is a preferred container. Capillary tubes are commercially available from a number of sources, including Polymicro Technologies, Inc., Phoenix, AZ. The capillary tube is preferably coated with a polymer, such as polyimide, that is mechanically stable. The coating must be removed in the region to be irradiated by the light source. An excimer laser can be used to remove the polymer coating. Preferably, at least 10 capillary tubes are used. More preferably, at least 96 capillary tubes are used.

The DNA samples and the control DNA samples are heated independently of the electrical field on the electrophoresis system through at least one predetermined temperature range selected to denature the DNA samples. If desired, at least two predetermined temperatures ranges can be selected. The

temperature ranges can be implemented as a function of time (i.e., temporal (°C/min)) or position (i.e., spatial (°C/cm)) along the container, e.g., capillary tube (see, e.g., Whang et al., Anal. Chem. 64: 502-506 (1992)). Desirably, the heating is carried out by a temperature program having a ramp of 0.2 to about 1.0 °C, preferably about 0.5 °C, at a precision that is about one-tenth of the ramp, preferably about 0.02 °C per step.

In many situations, the sequence of the target DNA fragment is already known. The position and type of mutation is either known or unknown. A melt map of the target DNA fragment can facilitate the determination of the optimal temperature range for discrimination. A melt map can be generated in accordance with methods known in the art. If the mutations occur at low-melt temperatures, a temperature range of 10 °C can be sufficient to detect the presence of mutations. However, if the mutations occur at high-melt temperatures, a broader temperature range must be applied. For an unknown mutation sample, i.e., where the sequence of the target DNA fragment is unknown, a sufficiently broad temperature range must be utilized to allow resolution. Alternatively, two or more separate temperature gradient runs can be utilized to cover the potential temperature range. A preferred predetermined temperature range is about 55 °C to about 75 °C.

Any suitable means can be used to heat the sample and the reference. A preferred means is exemplified in the Example herein. If ethidium bromide is used as the detectable label, the signal drops at high temperatures probably due to partial melting of the dsDNA and possibly weaker binding between DNA and the dye at high temperature. Thus, a sufficient amount of time for renaturation before detection needs to be allowed. While, the unheated part of the container after the heating plate may help recovery of the signal to some degree, it is desirable to increase the length of the unheated part of the container to provide for more time. For example, when a capillary tube is used as the container, an increase in length of 10 cm or so is sufficient.

Desirably, the containers are immersed in a high thermal conducter, which is in thermal contact with a heating source used to ramp the DNA samples and, when present, the control DNA samples, through the at least one predetermined spatial or temporal temperature range. Preferably, the high thermal conducter is in the form of a paste.

Detection employs an imaging means. The imaging means can be any suitable imaging means. For fluorescence detection, preferably, the imaging means is an intensified charge-coupled device (CCD) camera. The system can further comprise a microscope objective, such as a microscope objective of 10 x

power, between the container and the imaging means; the microscope object focuses the fluorescence from the fluorescent label onto the imaging means.

The absorbance of the DNA samples and, when present, the control DNA samples can be assessed. Any suitable means can be used to detect absorption. Preferably, the detection means comprises a plurality of absorption detection elements, such as a plurality of photosensitive elements, which desirably are positioned in a linear array, although a two-dimensional image array detector can be used. Desirably, the detection means is parallel to and in line with the multiple containers, which desirably are positioned in a linear array. Desirably, the detection means is rigidly mounted to reduce flicker noise. In this regard, the relative positions of cell components used in the system must be fixed.

A linear photodiode array (PDA) can be used. The PDA incorporates a linear image sensor chip, a driver/amplifier circuit and a temperature controller, which desirably thermoelectrically cools the sensor chip to a temperature from about 0 °C to about -40 °C. Lowering the temperature lowers the dark count and minimizes the temperature drift, thus enabling reliable measurements to be made over a wide dynamic range. The driver/amplifier circuit is desirably interfaced to a computer via an I/O board, which preferably also serves as a pulse generator to provide a master clock pulse and a master start pulse, which are required by the linear image sensor. The PDA records the image linearly -- not two-dimensionally. Preferably, the data acquired are written directly to the hard disk in real time. Also, preferably, the signals from up to at least about ten elements of the PDA are displayed in real time.

Alternatively, a CCD or a charge-injection device (CID) can be used. However, the CCD records in two dimensions, which is less efficient, requiring more computer memory, is slower, and requires every location to be read (not a single line like PDA). Furthermore, whereas a CCD has only 100,000 electrons in each location, each element in a PDA can store 59 million electrons per pixel per location; thus, given that detection sensitivity is related to the square root of the number of electrons that can be detected, a PDA is orders of magnitude more sensitive than a CCD.

Preferably, the PDA comprises linearly aligned pixels, in which case each container, such as each container in a planar array of multiple containers, desirably is a capillary tube and each capillary tube preferably is optically coupled to less than about ten pixels, more preferably from about 7 to about 9 pixels, some of which are coupled to the walls of the capillary tube and some of which are coupled to any space between the walls of adjacent capillaries and at least one of which is

coupled to the lumen of the capillary tube. Thus, the stray light caused by the walls of a capillary is dispersed prior to striking the pixels and/or is confined to the pixels coupled to the side walls and generally does not affect the signal produced by the pixel coupled to the lumen of the capillary. While the ratio of capillaries to optically coupled pixels is preferably less than about 1:10, more preferably from about 1:7 to about 1:9, the ratio of capillaries to optically coupled pixels need not be an integer ratio.

Desirably, the assaying is conducted in a light-tight construct, such as a metal box attached to an optical table. Also, desirably, the components are centered above the optical table.

The method can be carried out at ambient temperature, such as room temperature, such as from about 20 °C to about 30 °C, or as low as 0 °C or as high as 80 °C. However, if the method employs a PDA as the detection means, desirably the PDA has its own cooler for operation at subzero temperatures, such as from about 0 °C to about -40 °C.

The light source preferably comprises or consists essentially of a wavelength in the range from about 180 nm to about 1500 nm. Examples of suitable light sources include mercury (for ultraviolet (UV) light absorption), tungsten (for visible light absorption), iodine (for UV light absorption), zinc (for UV light absorption), cadmium (for UV light absorption), xenon (for UV light absorption), deuterium (for visible light absorption), and the like. Desirably, the light source comprises or consists essentially of a wavelength of light that will be absorbed by an absorbing species, the absorption of which is to be detected. Which wavelength of light will be absorbed by an absorbing species can be determined by a standard absorption spectrometer. Alternatively, spectroscopic tables that provide such information are available in the art, such as through the National Institute of Science and Technology (NIST). Desirably, a maximally absorbed wavelength of light is selected for a given absorbing species to be detected.

Generally, the light source provides light impinging on the multiple containers. The light source can be a point source. Also preferably, the light source has a power output of about 0.50 mW to about 50 mW. The light source can be AC or DC, although DC is preferred. Any flicker noise from the light source can be eliminated by using a double beam of light.

An optical filter desirably is positioned between the containers, such as a planar array of containers, and the detection means. The optical filter selects at least one wavelength of light from the light source that is absorbed by an

absorbing species, the absorption of which is to be detected. While an optical filter can be positioned between the light source and the multiple containers in addition to, or as an alternative to, an optical filter positioned between the multiple containers and the detection means, the placement of a single optical filter between the light source and the multiple containers is disadvantageous inasmuch as it does not block the subsequent fluorescence by the sample from reaching the detection means. In contrast, the placement of an optical filter between the multiple containers and the detection means blocks sample fluorescence from reaching the detection means.

A flat-field lens also desirably is positioned between the containers, such as a planar array of containers, and the detection means. The flat-field lens couples light that is not absorbed by the DNA in each sample in the containers with the detection means. While a lens that is not a flat-field lens can be used in the context of the present invention, it is disadvantageous if it does not image the entire field evenly. Consequently, the edges of the containers positioned at the edges of the field of the lens cannot be detected or measured. The lens inverts the image of the planar array of multiple containers onto the face of the detection means, which preferably is a PDA.

Desirably, the coupling of light by the flat-field lens is shielded from the light source. This way, only the light from the lens is focused on the detection means.

A beam expander can be positioned between the light source and the containers, such as a planar array of containers. The beam expander can alter the focused line of the light source so as to irradiate more effectively the multiple containers. The beam, optionally, can be altered or redirected, as with a mirror, filter or lens, prior to contacting the containers.

A collimating focusing lens can be positioned between the light source and the multiple containers. In addition, the components are placed to eliminate substantially and, desirably, completely, stray light.

Preferably, raw data sets are extracted into single-diode electropherograms and analyzed by converting the transmitted light intensities collected at the PDA to absorbance values. Root-mean-squared noise in the electropherograms is obtained using a section of baseline near one of the analyte peaks. Mathematical smoothing can be used to reduce the noise significantly, without distorting the signal.

The resulting electropherograms of the heteroduplexed DNA and homoduplexed DNA are compared so as to detect the presence of a mutation in the DNA fragment sample. Even a slight pattern change is sufficient to recognize a

mutation, since this is a highly reproducible system. Perfect separation of the fragments in heteroduplex is not necessary. The pattern change can be determined by the existence of additional peaks (at most four total), peak shoulders or even broader peak width. Additional peaks can be quite obvious and can lead to the identification of mutations with a high level of confidence. Peak shoulders and peak width, however, can lead to the identification of false positives. However, a false positive is less of a concern than a false negative in diagnosis, since further tests, such as sequencing, will be performed in such situations. The actual confidence level can be determined from the 2% relative standard deviation for the migration times and the level of the pattern change derived from curve fitting.

EXAMPLE

The following example serves to illustrate the present invention and is not intended to limit the scope of the present invention.

TBE buffer (1x) was prepared by dissolving 8.5 g premixed TBE buffer powder (Amerosco, Solon, OH) into 500 ml of deionized water. Ethidium bromide was incorporated into the TBE buffer with a final concentration of 0.5 μg/ml.

Poly(vinylpyrrolidone) (PVP) was obtained from Sigma Chemical Co. (St. Louis, MO). The sieving matrix was made by dissolving 3% (w/v) of 360,000 M_w PVP into 1x TBE buffer with dye. The mixture was shaken for 2 min and left standing for 1 hr to remove bubbles.

The point mutation samples and their references were provided by Dr. Peter Oefner and Dr. Peidong Shen of Stanford University. The characteristics of the DNA samples were as follows:

Table 1

Characteristics of the DNA Samples

Sample	Length (bp)	Mutation Type	Mutation Position	Primer Sequence
M_2	209	A to G	169	Forward = AGGCACTGGTCAGAATGAAG Reverse = AATGGAAAATACAGCTCCCC
M_{60}	388	T insertion	243	Forward = GCACTGGCGTTCATCATCT Reverse = ATGTTCATTATGGTTCAGGAGG
M_{69}	256	T to C	221	Forward = GGTTATCATAGCCCACTATACTTTG Reverse = ATCTTTATTCCCTTTGTCTTGCT
$M_{122(a)}$	393	T to C	73	Forward = TGGTAAACTCTACTTAGTTGCCTTT Reverse = CAGCGAATTAGATTTTCTTGC
$M_{122(b)}$	393	T to C	289	Forward = TGGTAAACTCTACTTAGTTGCCTTT Reverse = CAGCGAATTAGATTTTCTTGC

The heteroduplex was formed by mixing, denaturing, and then reannealing the reference sample and mutant at a ratio of 1:1. The melt map of the samples was constructed with a computer program Melt 94 (Lerman et al., *Mutation Detection: A Practical Approach*; Oxford University Press: New York, 1998; pp. 45-50.).

A laboratory assembled capillary electrophoresis system with laser-induced fluorescence detection and a heating plate (48 cm x 10 cm x 0.8 cm aluminum plate with 300 W heating element and platinum sensor closely attached underneath) was used as shown in Fig. 1, which is a diagram of a system for use in the present inventive method. An 84 cm capillary with 375 μm o.d. and 75 μm i.d. (Polymicro Technologies, Phoenix, AZ) was attached to the heating plate. There was an 18 cm length of capillary in front of and after the heating plate. The capillary was immersed into a layer of high-thermal-conductive paste on the heating plate for better temperature control as shown in Fig. 2, which is a diagram of the heating device for temperature-gradient capillary electrophoresis. The temperature was regulated and monitored by an Eppendorf TC-50 temperature controller (Brinkmann Instruments, Westbury, NY). The temperature controller was modified in-house so that it can be programmed by a computer through

LabView software. A temperature setting from 58-78 °C with a precision of 0.02 °C per step was possible. Since the capillary has a small volume and excellent contact with the heating plate through the thermal-conductive paste, the temperature inside the capillary will follow the temperature reading of the plate closely at these ramp conditions. A 514 nm Ar^+ laser was used for excitation. The applied laser power was 5 mW. A PMT with a 590 nm long pass filter was used for collecting the flourescence. The sampling rate was 5 Hz. See, for example, Gao et al., Anal. Chem. 70: 1382-1390 (1998).

The capillary was filled with 3% PVP solution without any pretreatment. The temperature was preset to 61 °C before injection. Sample injection was conducted at a field strength of 220 V/cm for 10 seconds. The temperature program was selected as follows: 4 min delay after injection, 0.7 °C/min ramp at a precision of 0.02 °C per step. The gradient terminated at 71 °C. Then the heating plate was returned to 61 °C. After each run, fresh PVP matrix was used to simply flush out the old one to maintain the separation efficiency in the subsequent run. During idle periods, the capillary array was stored in deionized water. The same capillaries were used over a period of two months without showing any signs of degradation.

The instrumentation was similar to one reported earlier (Gao et al. (1999), *supra*). A total of 96 capillaries (75 μm i.d., 15 μm o.d. (Polymicro)) was packed side-by-side at the detection windows. An Ar^+ ion laser (Coherent I-90) with 80 mW output at 514 nm was used for excitation. A 5 cm focal length cylindrical lens was used to expand the laser beam to approximately 4 cm upon the detection window to cover the entire array. Another 10-cm focal length cylindrical lens was used to focus the laser beam vertically on the detection windows. A CCD camera (Princeton Instruments, Inc., model TE/CCD-512-TKB) with a 28 mm Nikon camera lens was used for image capture. The CCD was arranged in a way such that approximately 300 pixels were used to view the 1.5 cm detection region. In order to simplify alignment, a 5 x 300 pixel image was used on the detection windows to ensure all capillaries were covered. Winspec software (Version 1.3B) from Princeton Instruments was used to control the CCD camera. A holographic notch filter (Kaiser Optical System) was located in front of the CCD chip to filter out the laser scatter. At the ground end for the capillary array, the capillaries were bundled together to allow simultaneously matrix filling. At the sample injection end, the capillary array was spread out and mounted on a copper plate to form an 8 x 12 format with dimensions that fit a 96-well microtiter plate for sample injection. In addition, 96 gold-coated pins (Mill-Max, Oyster Bay, NY) were

located next to the capillary tips to serve as electrodes. A high-voltage DC power supply from Glassman (Whitehorse Station, NJ) provided power for electrophoresis.

Excellent migration time and electrophoretic pattern reproducibility were observed from run to run with a test heteroduplexed DNA sample. The relative standard deviation of migration time was within 2%.

Since the mobility retardation occurred only when the DNA fragments began to melt, the part of the capillary that was not on the heating plate did not affect the mobility of the fragments. The time that the DNA fragment spent in the unheated parts of the capillary can be accounted for and the temperature gradient can be started once the DNA fragments migrate into the heated part. The mobility of DNA fragments in the unheated part can be calculated from running the sample at room temperature. An electric field correction should be made because the voltage drop along the capillary is not uniform when a part of a capillary is heated. Since the conductivity of the unheated part is independent of heating of the other parts, the electric field across it should be proportional to the current through it. By adjusting the current to the same value under heated condition, the DNA mobility measured at room temperature should be the same as that in the unheated part when the plate is heated. M_2 and M_{122} are the smallest and largest DNA fragment among the samples. It takes 4 min 30 sec and 4 min 50 sec, respectively, to migrate into the heated region after injection. The migration time for the rest of the samples will fall between these values. Considering the temperature ramp is 0.7 °C/min and the temperature range is 10 °C, the variation of temperature profiles among the samples is negligible. Alternatively, a spatial gradient could have been used.

The calculated melt maps (Tm (°C) vs. position (base pairs)) of the four samples, namely M2, M60, M69 and M122, of Table 1 are shown in Figs. 3A-3D, respectively. The marks correspond to the positions of single-point mutations. Each melt map shows the equilibrium temperature, i.e., Tm, along the sequence at which each base pair in the molecule has equal probability for the helical or melted states as long as the strands remain associated. The low-melt domain of all of the samples fall into the temperature range of 60-70 °C, and the mutation positions are all in the low-melt domain. Thus, a temperature range of 10 °C between 61 °C and 71 °C should be able to uncover all of the mismatches.

The electropherograms (intensity vs. time (min)) of the four samples of Table 1, namely M2, M60, M69 and M122 are shown in Figs. 4A-4D, respectively. Fast separations were achieved under the same temperature gradient,

i.e., 61-71 °C. Although the samples had different fragment lengths and different mutations, they all showed recognizable pattern differences between homoduplexed and heteroduplexed DNA. Occasionally, multiple peaks were observed for the homoduplexed DNA samples because of impurity and PCR problems. Given that this temperature gradient was not the best for all of the samples (i.e., the mutation in the M_{60} sample is obvious in Fig. 4B but the four components are not completely resolved), the experiment was repeated for M_{60} at a temperature gradient from 68-74 °C and a ramp of 0.5 °C/min and again at a temperature gradient from 61-68 °C and a ramp of 0.5 °C/min. The results are shown in the electropherograms (intensity vs. time (min)) of Fig. 5A and Fig. 5B, respectively.

Using the instrumentation of Gao et al. (1999), *supra*, all of the samples, i.e., five heteroduplexed and four homoduplexed DNA fragments, were characterized simultaneously. The heating plate imposed an identical temperature gradient on all of the 96 capillaries. The heat capacity of each capillary was low so the result were similar to those for the single capillaries of Figs. 4A-4D. The results are shown in Figs. 6 and 7. Fig. 6 is a two-dimensional image (migration time vs. number of the capillary for time scale of 0-40 min) of the 96 electropherograms for detection of mutations in the five heteroduplexed and four homoduplexed DNA fragments. Fig. 7 represents extracted electropherograms (intensity vs. time (sec)), one for each of the mutation samples studied, from Fig. 6. Accordingly, high-speed, high-throughput screening for unknown mutations, particularly SNPs, is possible in accordance with the present invention.

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method for detecting the presence of mutations in DNA samples by multiplexed electrophoresis, which method comprises:

(a) introducing each of the DNA samples, all of which comprise a buffer solution comprising a matrix and at least one of which comprises heteroduplexed DNA, separately into a plurality of containers in an electrophoresis system;

(b) heating the DNA samples independently of the electrical field in the electrophoresis system through at least one predetermined spatial or temporal temperature range selected to denature the DNA samples; and

(c) detecting the presence of any mutations in the DNA samples by comparing an electrophoretic pattern for each of the DNA samples with an electrophoretic pattern for a control DNA sample, whereupon the presence of mutations in DNA samples is detected by multiplexed electrophoresis.

2. The method of claim 1, wherein the method further comprises in (b) introducing at least one control DNA sample, which comprises a homoduplexed DNA in a buffer solution comprising a matrix, separately into a container in the plurality of containers in the electrophoresis system.

3. The method of claim 1 or 2, wherein the at least one buffer solution further comprises a fluorophore and said detecting includes irradiating the DNA samples and, when present, the control samples with a coherent light to cause fluorescence and the detecting includes determining the fluorescence of the DNA samples and, when present, the control samples.

4. The method of claim 3, wherein the fluorophore increases the rigidity of the DNA.

5. The method of claim 3 or 4, wherein the fluorophore is ethidium bromide.

6. The method of claim 1 or 2, wherein the detecting includes determining the absorbance of the DNA samples and, when present, the control samples.

7. The method of any of claims 1-6, wherein the matrix is poly(vinylpyrrolidone).

8. The method of any of claims 1-7, wherein the at least one predetermined temperature range is about 55 °C to about 75 °C.

9. The method of any of claims 1-8, wherein, in step (b), the DNA samples and, when present, the control DNA samples are heated independently of the electrical field in the electrophoresis system through at least two predetermined temperature ranges selected to denature the DNA samples.

10. The method of any of claims 1-9, wherein the heating is carried out by a temperature program having a ramp of from 0.2 °C to about 1.0 °C at a precision of about one-tenth of the ramp.

11. The method of any of claims 1-10, wherein the heating is carried out by a temperature program having a ramp of about 0.5 °C or more at a precision of about 0.02 °C or more per step.

12. The method of any of claims 1-5 and 7-11, wherein the detecting is carried out using a charge-coupled-device-based imaging system.

13. The method of any of claims 1-12, wherein the containers are immersed in a high thermal conductor, which is in thermal contact with a heating source used to ramp the DNA samples through the at least one predetermined temperature range.

14. The method of claim 13, wherein the high thermal conductor is in the form of a paste.

15. The method of any of claims 1-14, wherein the containers are capillary tubes.

16. The method of claim 15, wherein there are at least 10 capillary tubes.

17. The method of claim 16, wherein there are at least 96 capillary tubes.

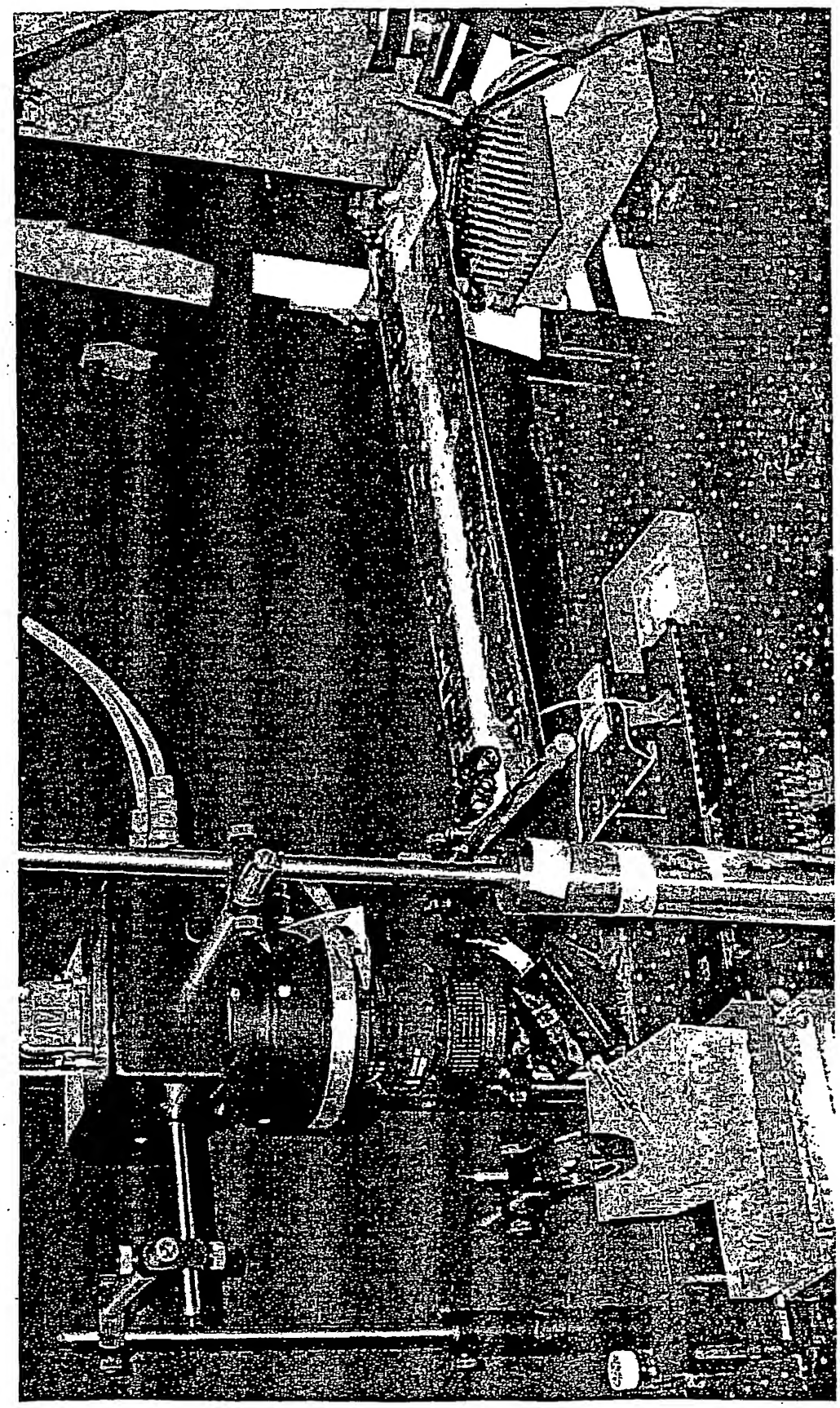

FIG. 1

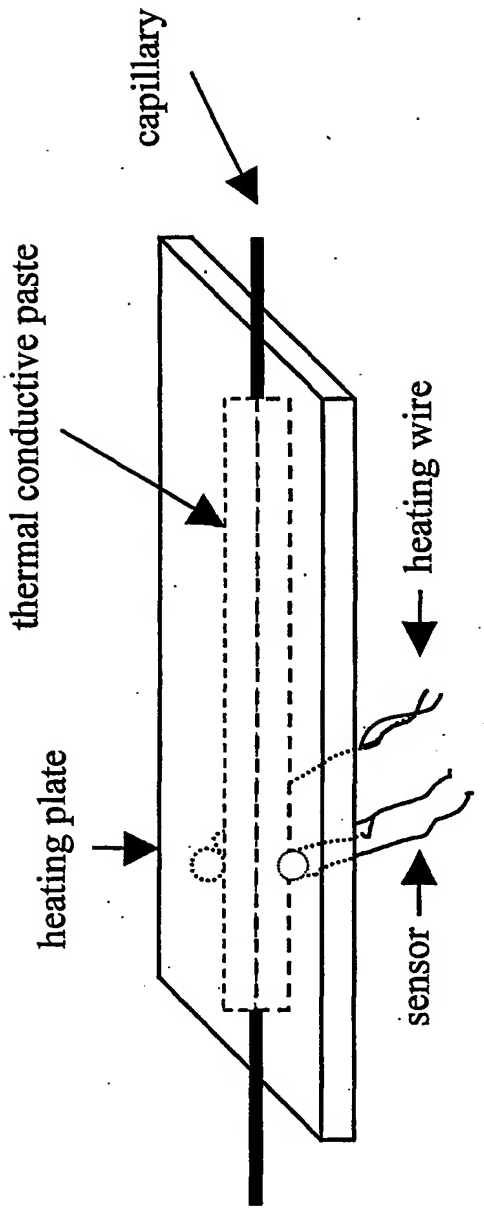
capillary
thermal conductive paste
heating wire
heating plate
sensor

FIG. 2

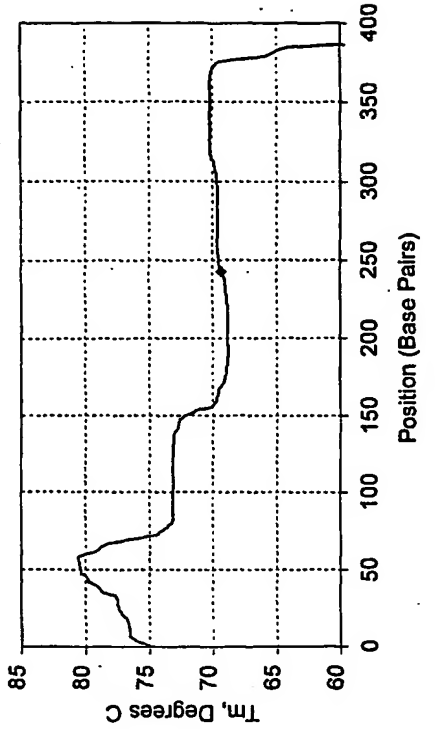

FIG. 3B

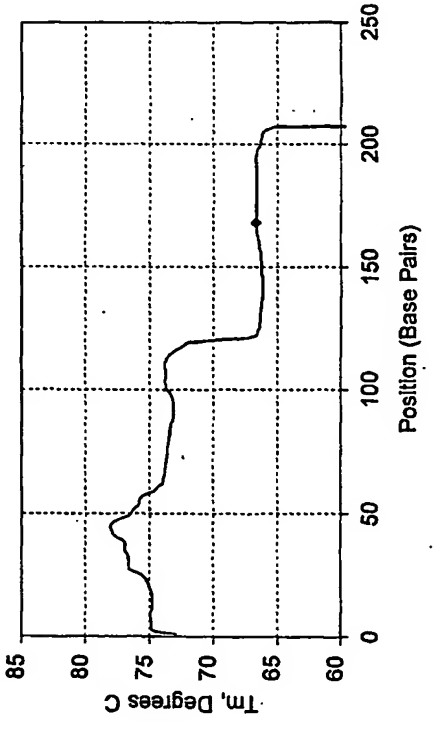

FIG. 3A

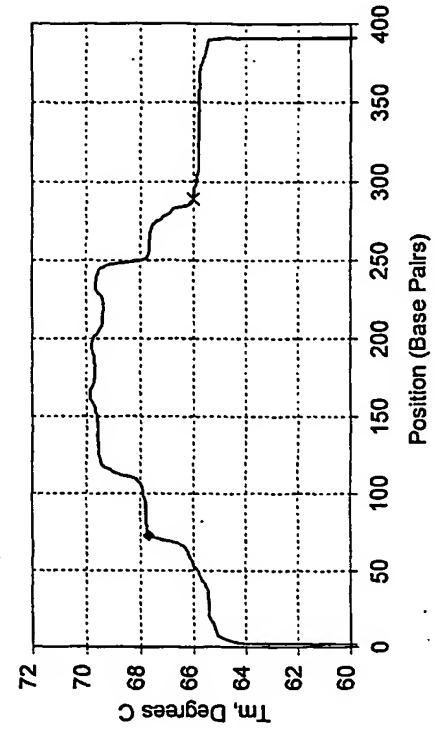

FIG. 3D

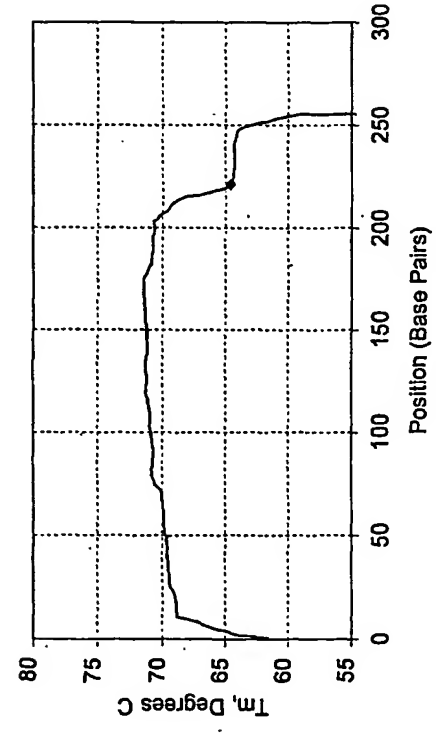

FIG. 3C

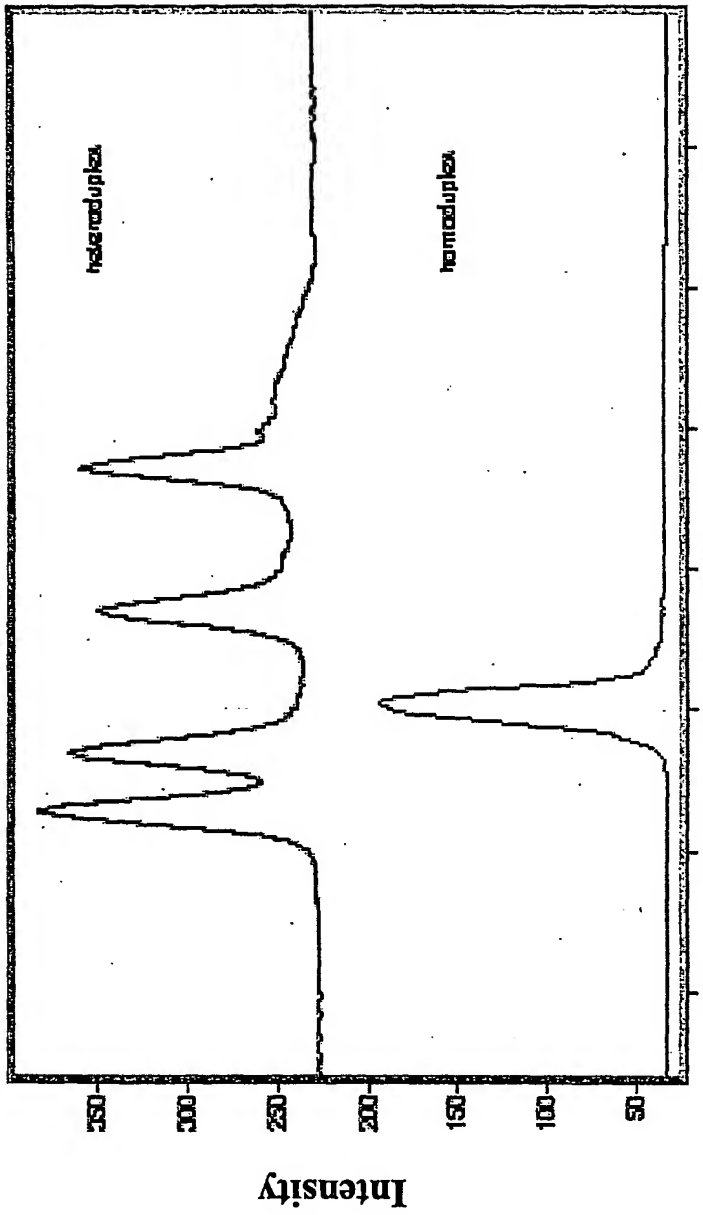
heteroduplex
homoduplex
Intensity
250
200
150
100
50
17
17.5
18
18.5
19
19.5
20
Time (min)

FIG. 4A

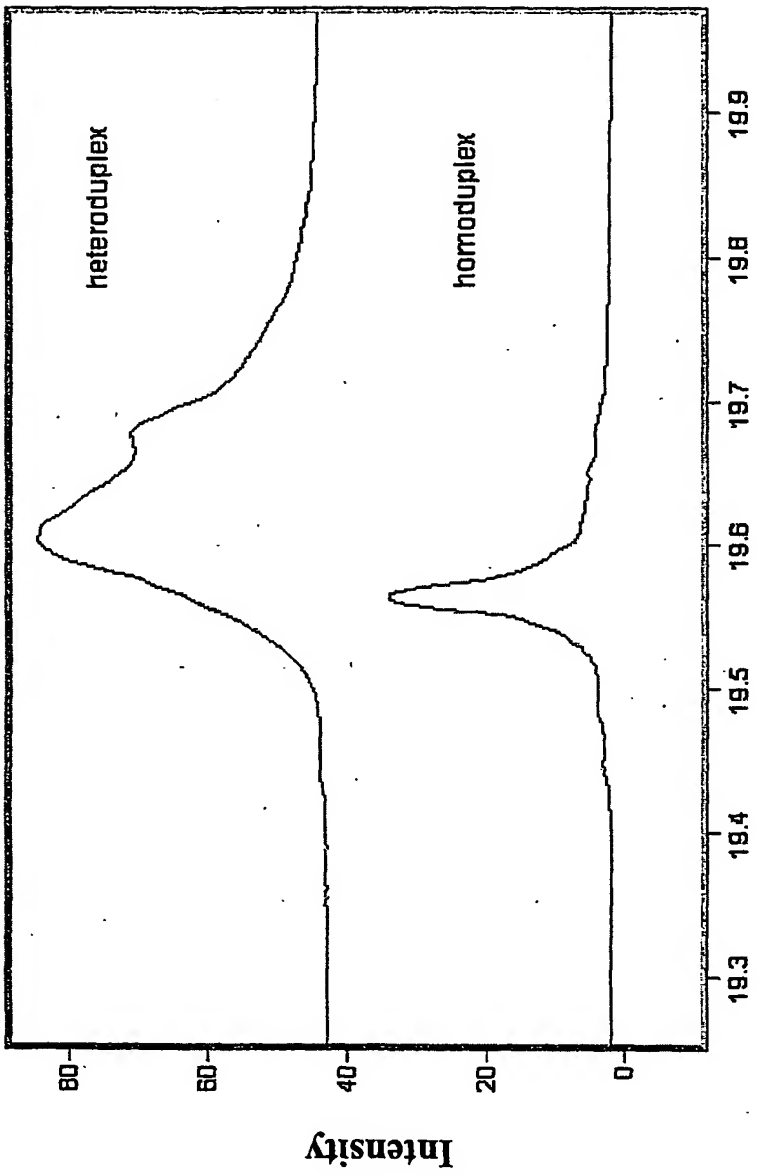
heteroduplex
homoduplex
Intensity
80
60
40
20
0
19.3
19.4
19.5
19.6
19.7
19.8
19.9
Time (min)

FIG. 4B

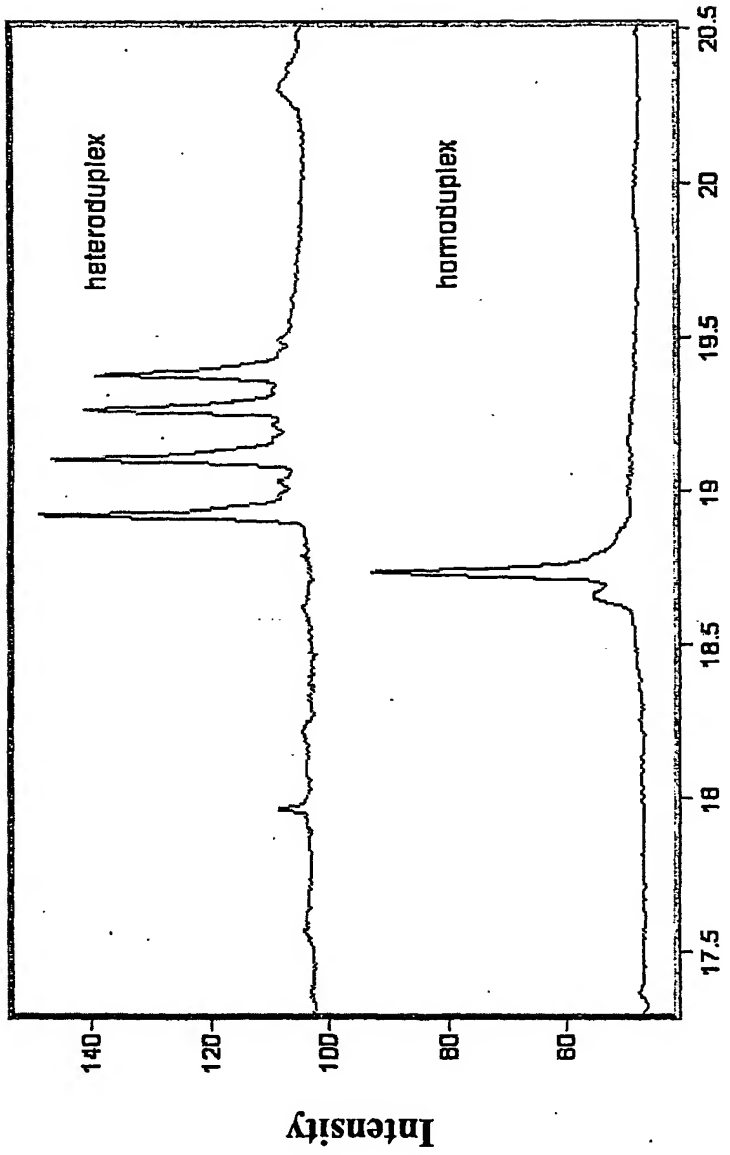
heteroduplex
homoduplex
Intensity
140
120
100
80
60
17.5
18
18.5
19
19.5
20
20.5
Time (min)

FIG. 4C

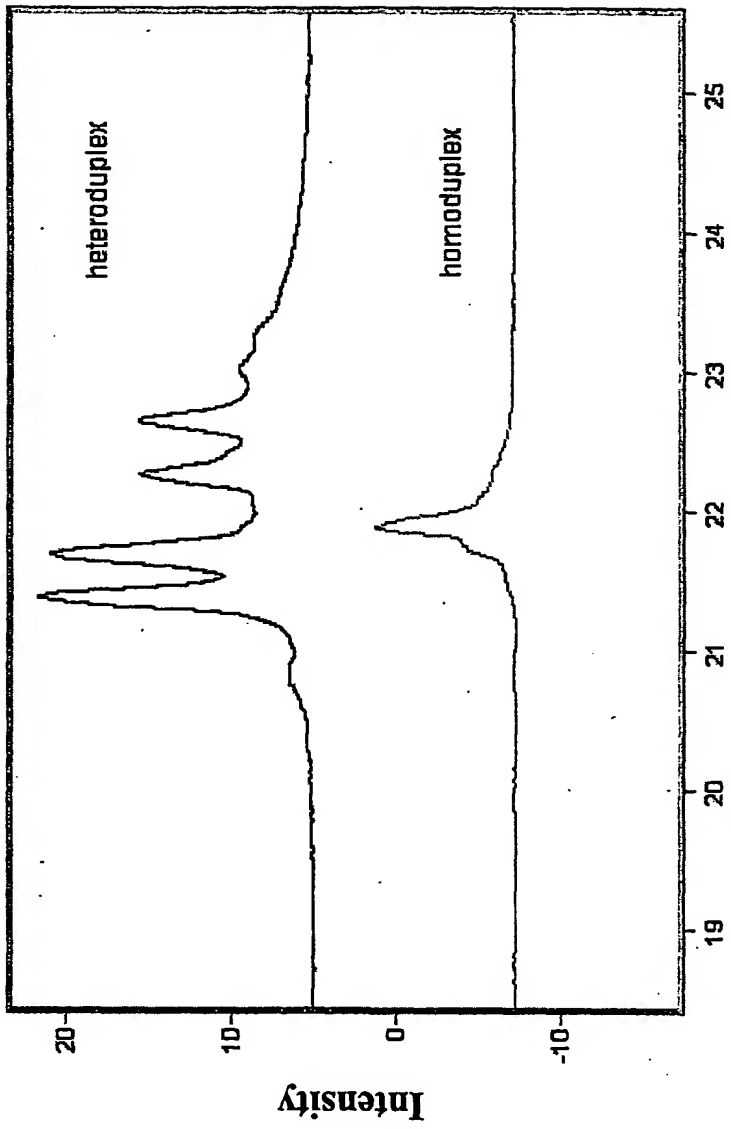
heteroduplex
homoduplex
Intensity
20
10
0
-10
19
20
21
22
23
24
25
Time (min)

FIG. 4D

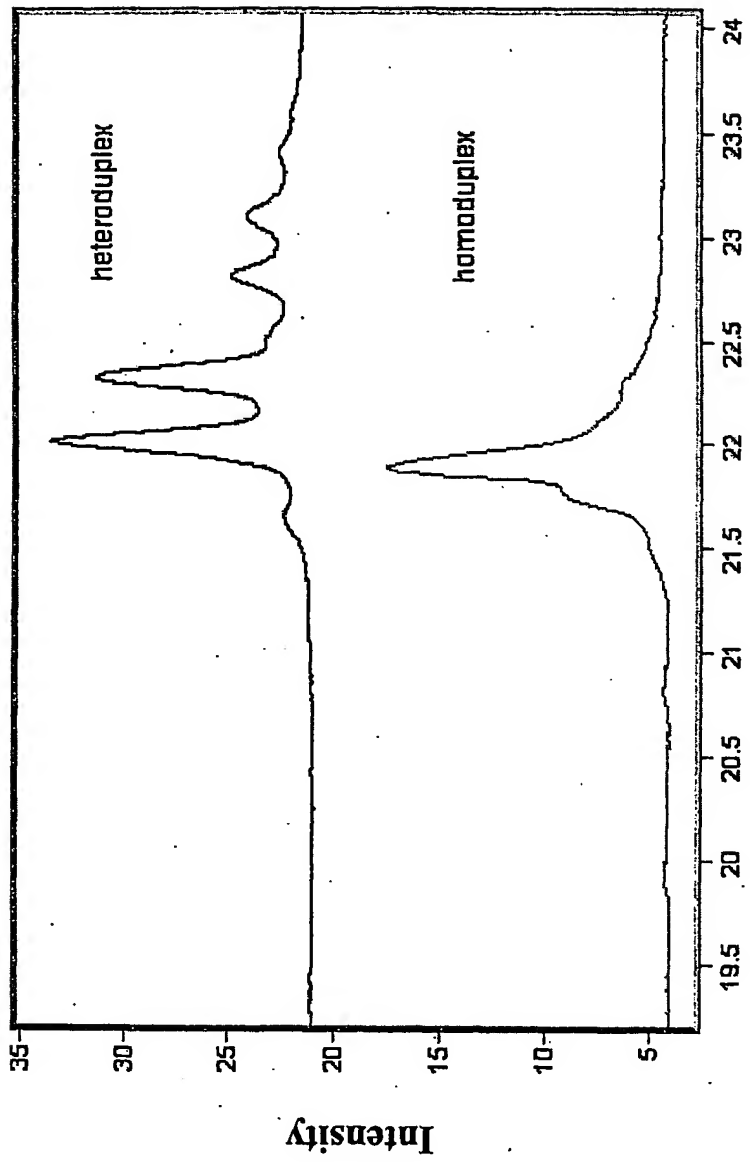
heteroduplex
homoduplex
Intensity
35
30
25
20
15
10
5
19.5
20
20.5
21
21.5
22
22.5
23
23.5
24
Time (min)

FIG. 4E

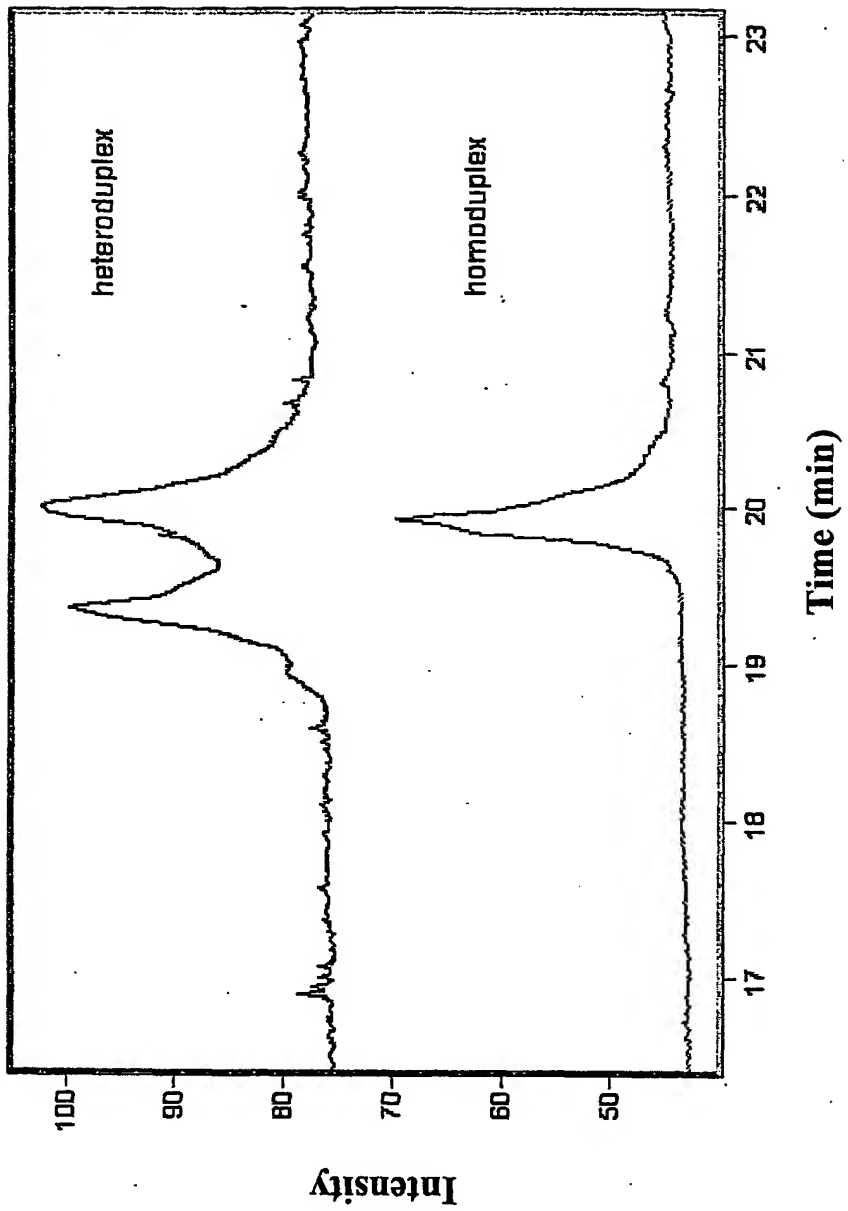
heteroduplex
homoduplex
Intensity
100
90
80
70
60
50
17
18
19
20
21
22
23
Time (min)

FIG. 5A

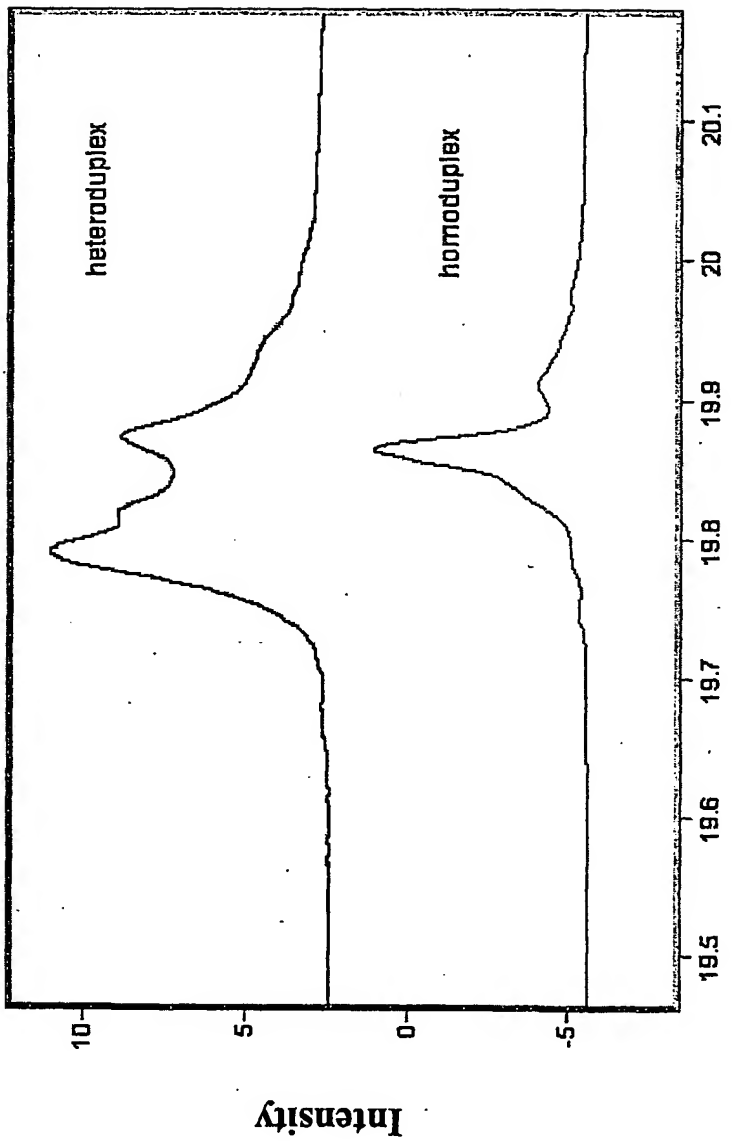
heteroduplex
homoduplex
10
5
0
-5
19.5
19.6
19.7
19.8
19.9
20
20.1
Time (min)
Intensity

FIG. 5B

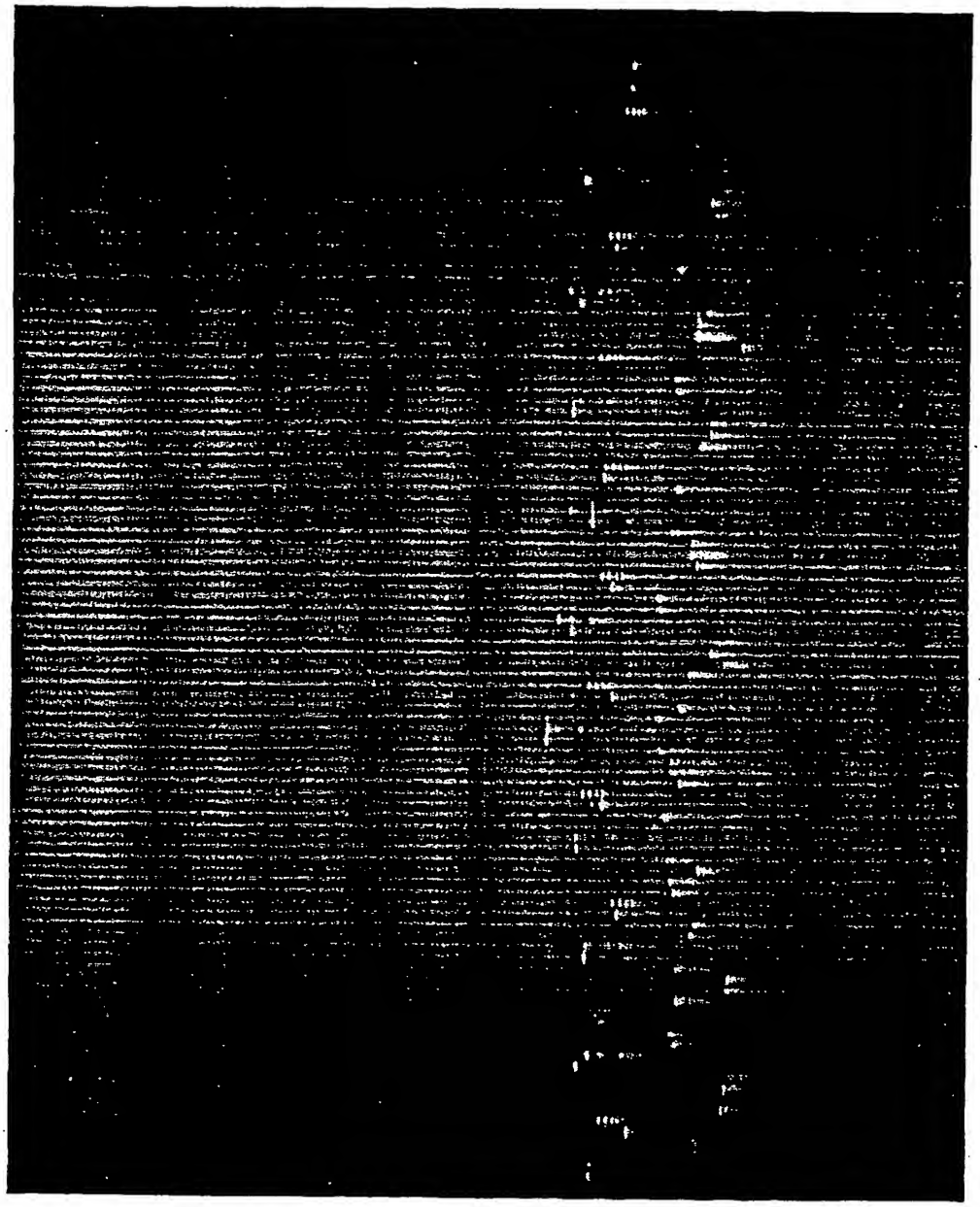

FIG. 6

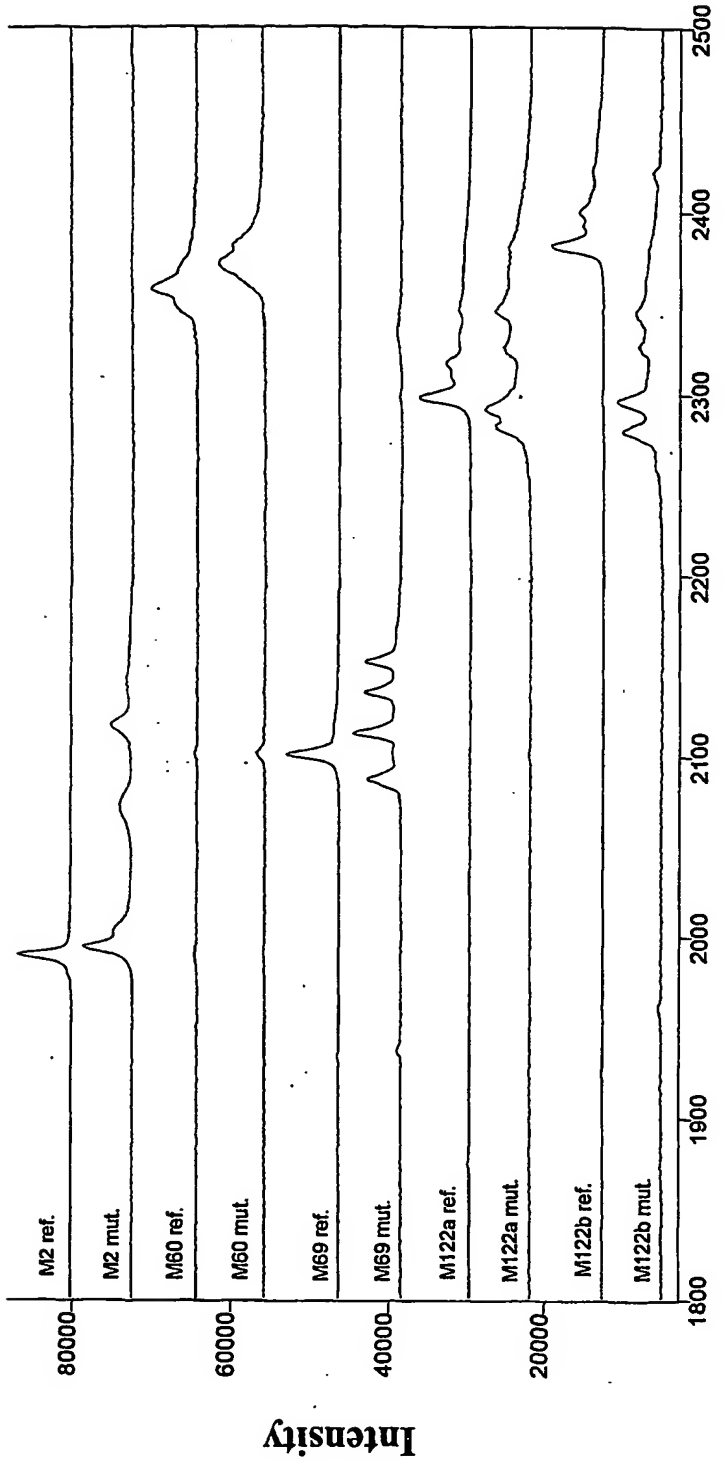
M2 ref.
M2 mut.
M60 ref.
M60 mut.
M69 ref.
M69 mut.
M122a ref.
M122a mut.
M122b ref.
M122b mut.
80000
60000
40000
20000
1800
1900
2000
2100
2200
2300
2400
2500
Intensity
Time (s)

FIG. 7

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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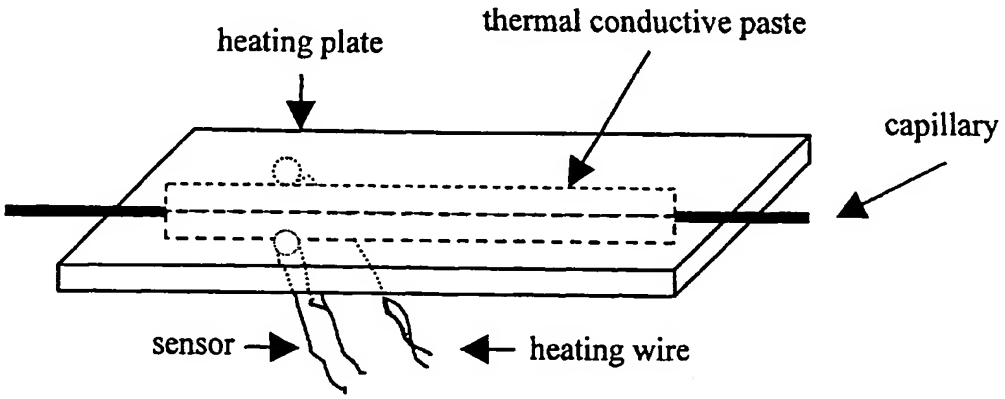
23 January 2003

[Continued on next page]

(54) Title: HIGH-THROUGHPUT DETECTION OF UNKNOWN DNA MUTATIONS BY USING MULTIPLEXED CAPILLARY ELECTROPHORESIS



(57) Abstract: A method for detecting the presence of mutations in DNA samples by multiplexed electrophoresis, which method comprises: (a) introducing each of the DNA samples, all of which comprise a buffer solution comprising a matrix and at least one of which comprises heteroduplexed DNA, separately into a plurality of containers in an electrophoresis system; (b) heating the DNA samples independently of the electrical field in the electrophoresis system through at least one predetermined spatial or temporal temperature range selected to denature the DNA samples; and (c) detecting the presence of any mutations in the DNA samples by comparing an electrophoretic pattern for each of the DNA samples with an electrophoretic pattern for a control DNA sample, whereupon the presence of mutations in DNA samples is detected by multiplexed electrophoresis. The method can further comprise in (b) introducing at least one control DNA sample, which comprises a homoduplexed DNA in a buffer solution comprising a matrix, separately into a container in the plurality of containers in the electrophoresis system.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/10893

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 24687 A (UNIV LONDON ;DAY IAN NICHOLAS MONSARRATT (GB)) 15 August 1996 (1996-08-15) the whole document	1-17
Y	WO 91 02815 A (DIAGEN INST MOLEKULARBIO) 7 March 1991 (1991-03-07) the whole document	1-17
Y	US 5 066 377 A (RIESNER DETLEV ET AL) 19 November 1991 (1991-11-19) see abstract	1-17

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[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2002	05/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/10893

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	GONG X ET AL: "Genetic typing and HIV-1 diagnosis by using 96 capillary array electrophoresis and ultraviolet absorption detection" JOURNAL OF CHROMATOGRAPHY. BIOMEDICAL APPLICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 741, no. 1, April 2000 (2000-04), pages 15-21, XP004197021 ISSN: 0378-4347 page Y	1-17
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/10893

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